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<b>(21) International Application Number: PCT/US97/20227</b> <b>(22) International Filing Date: 12 November 1997 (12.11.97)</b>  <b>(30) Priority Data:</b> 08/748,591                      13 November 1996 (13.11.96)    US  <b>(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US];</b> 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).  <b>(72) Inventors: EPSTEIN, Ervin;</b> 533A Miner Road, Orinda, CA 94563 (US). HU, Zhiljan; 742 Judah Street, San Francisco, CA 94110 (US). BONIFAS, Jeanette; 252 Andover Street, San Francisco, CA 94110 (US).  <b>(74) Agent: SHERWOOD, Pamela, J.; Bozicevic &amp; Reed LLP, Suite 200,</b> 285 Hamilton Avenue, Palo Alto, CA 94301 (US).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title: MUTANT HUMAN HEDGEHOG GENE</b>		
<b>(57) Abstract</b> <p>A mutation in the human <i>sonic hedgehog</i> gene is associated with tumorigenesis. A variety of human tumors, including basal cell carcinomas, breast carcinomas, medulloblastomas, etc., have a somatic mutation that results in an amino acid substitution at position 133 [*his133 SHH], or in a mutation at position 114. Such mutated genes and fragments thereof, encoded protein, and antibodies specific for the mutated protein are useful in characterizing the phenotype of associated tumors. The mutant protein is useful in drug screening for compositions that antagonize or otherwise modulate HH activity or expression. The encoded protein is also used as a therapeutic, to modulate cell proliferation and differentiation, and treatment of pathological conditions associated with decreased hedgehog signaling.</p>		

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## MUTANT HUMAN HEDGEHOG GENE

## STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with Government support under Grant no. AR39959, awarded by the National Institutes of Health. The Government has certain rights in this  
5 invention.

## INTRODUCTION

Life requires balance. At all levels, from populations, individuals, cells, and even down to biochemical signals within a cell, there is a balance of opposing forces. Replication is a particularly important process to keep under tight control, with cancer  
10 serving as an archetype for uncontrolled cell growth. Unraveling both sides of the biochemical signals that regulate growth has provided insight into the mechanisms of tumor cell transformation.

Many genes involved in the regulation of growth and control of cellular signaling are also involved in oncogenesis. Such genes may be oncogenes, which are  
15 typically upregulated in tumor cells, or tumor suppressor genes, which are down-regulated or absent in tumor cells. Malignancies can arise when a tumor suppressor is lost and/or an oncogene is inappropriately activated. Information about these regulatory signals can be used in cancer diagnosis and treatment. This knowledge can also be exploited to counteract situations where there is inadequate cell growth.

20 Segment polarity genes were originally discovered as mutations in flies that change the pattern of body segment structures. Mutations in these genes cause animals to develop changed patterns on the surfaces of body segments; the changes affecting the pattern along the head to tail axis. Among the genes in this class are *hedgehog*, which encodes a secreted protein (HH), and *patched* (PTC), which encodes a protein structurally  
25 similar to transporter proteins. The *hedgehog* gene of flies has at least three vertebrate relatives: *Sonic hedgehog* (SHH); *Indian hedgehog* (IHH), and *Desert hedgehog* (DHH). Based on genetic experiments in flies, *patched* and *hedgehog* have antagonistic effects in development. PTC down regulates transcription of itself, members of the transforming growth factor  $\beta$  and *Wnt* gene families, and possibly other genes. Among other activities,

HH up regulates expression of *patched* and other genes that are negatively regulated by *patched*.

- Recently, interest in the human *hedgehog* signaling pathway was piqued by the discovery of inherited *patched* gene mutations in patients with basal cell nevus syndrome, a rare autosomal dominant disease characterized by developmental abnormalities and frequent skin carcinomas. Somatic acquired mutations of the *patched* gene have been identified in sporadic cancers, including basal cell carcinomas, primary breast carcinomas, medulloblastomas and meningiomas. It is currently believed that *patched* acts as a tumor suppressor, and that these mutations cause a loss of function in the *patched* gene product.
- 10 The *hedgehog/patched* signaling pathway may therefore be a factor in tumorigenesis.

Detecting genetic alterations that lead to increased cell growth and tumorigenesis is of great interest for clinical medicine. Identifying the specific changes that lead to altered cell growth may open the door to improved diagnosis and possible treatment for associated tumors.

#### 15 *Relevant Literature*

- Discussion of the role of *hedgehog* may be found in Riddle *et al.* (1993) Cell 75:1401-1416; Echelard *et al.* (1993) Cell 75:1417-1430; Krauss *et al.* (1993) Cell 75:1431-1444 (1993); Tabata and Kornberg (1994) Cell 76:89-102; Heemskerk and DiNardo (1994) Cell 76:449-460; and Roelink *et al.* (1994) Cell 76:761-775.
- 20 Marigo *et al.* (1995) Genomics 28:44-51 provide gene sequences for the wild-type human *hedgehog* genes. *Hedgehog* proteins are synthesized as approximately 300 amino acid precursors. An amino terminal signal peptide is released, and the remaining protein is auto-catalytically cleaved to generate a membrane bound N-terminal portion and a soluble C-terminal portion (Porter *et al.* (1995) Nature 374:363-366). The N-terminal
- 25 portion, extending from approximately amino acid 26 to amino acid 256, appears to be the active fragment involved in signaling (Feitz *et al.* (1995) Current Biology 6:643-650). It is apparently bound to the membrane by a lipophilic cholesterol moiety attached to its cleaved C-terminus (Porter *et al.* (1996) Cell 86:21-34; Porter *et al.* (1996) Science 274:255-259). The crystal structure of the N-terminus suggests the presence of a potential
- 30 zinc hydrolase catalytic site (Hall *et al.* (1995) Nature 378:212-216).

Descriptions of *patched* gene function may be found in Hooper and Scott (1989) Cell 59:751-765; Nakano *et al.* (1989) Nature 341:508-513; and Goodrich *et al.* (1996) Genes Develop. 10:301-312. The association of *patched* mutations with basal cell nevus syndrome and sporadic tumors are described in Johnson *et al.* (1996) Science 272:1668-1671; Hahn *et al.* (1996) Cell 85:841-851; and Gailani *et al.* (1996) Nature Genetics 14:78-81.

#### SUMMARY OF THE INVENTION

Isolated nucleic acid compositions encoding mutated forms of human hedgehog genes are provided. The mutated form of the sonic hedgehog (SHH) protein product is associated with oncogenic transformation. The nucleic acid compositions, and antibodies specific for the mutated protein product are useful as diagnostics to identify HH-associated cancers. The nucleic acid compositions are also used to produce the encoded protein, which may be employed for functional mapping studies, as a therapeutic, and in studying associated physiological pathways. Down-regulation of the mutated gene activity *in vivo* is useful for therapeutic purposes.

#### DATABASE REFERENCES FOR NUCLEOTIDE AND AMINO ACID SEQUENCES

The complete mRNA sequence encoding human sonic hedgehog (SHH) has the Genbank accession number L38518. The gene is located on human chromosome 7q36, and has the genome database accession number GDB:456309. A partial sequence of human indian hedgehog (IHH) mRNA, 5' end, has the Genbank accession number L38517. The gene is located on human chromosome 2pter, and has the genome database accession number GDB:511203. A partial sequence of human desert hedgehog (hDHH) mRNA, has the Genbank Accession number U59748.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Human *hedgehog* gene and gene product compositions are provided. The genes encode a mutant form of *hedgehog* that is associated with tumorigenesis. The subject genes and fragments thereof, encoded protein, and antibodies specific for the mutated protein are useful in characterizing the phenotype of associated sporadic tumors.

Certain human tumors, *e.g.* basal cell carcinomas, breast carcinomas, medulloblastomas, *etc.*, have been found to have an oncogenic mutation in the *sonic hedgehog* gene that results in an amino acid substitution at position 133 [*\*his133 SHH*]. Tumors may be typed or staged by detection of mutated sequences, antibody detection of abnormal protein products, and functional assays for altered *hedgehog* activity. The encoded protein is useful in drug screening for compositions that antagonize or otherwise modulate HH activity or expression. The encoded protein is also used as a therapeutic, to modulate cell proliferation and differentiation, and treatment of pathological conditions associated with decreased hedgehog signaling.

As used herein, the term *hedgehog* (HH) genes and encoded proteins shall be used to generally designate any of the mammalian *hedgehog* genes and gene products, and unless otherwise stated will be the human homolog. Hedgehog genes may be characterized by sequence similarity to known hedgehog genes, and usually have at least about 75% sequence identity at the nucleotide, more usually at least about 85% sequence identity, and may have as much as 90% sequence identity. Methods for identifying genes by nucleic acid hybridization, or by using sequence comparison algorithms, are known in the art. Known members of the Hedgehog gene family include sonic, indian and desert hedgehog. Oncogenic mutations in HH are associated with the transformation of normal cells to tumor cells. Specific examples of oncogenic mutations are described below.

Hedgehog mutations that result in an amino acid substitution or deletion at the position corresponding to his133 in SHH, will be generally referred to as [*\*his HH*]. This amino acid position corresponds to position 50 of the previously referenced desert hedgehog protein (DHH), and position 39 of the previously referenced indian hedgehog protein (IHH). The alignment of amino acid sequences from the three hedgehog proteins is as follows, with the histidine residue underlined, and asterisks denoting identity between two sequences.

```
(DHH) SEQ ID NO:1  NALAIAMNMWPGVRLRVTEGWDEDGHHQAQ
                   *****
(SHH) SEQ ID NO:2  NALAISVMNQWPGVKLRVTEGWDEDGHHSE
                   * *****
(IHH) SEQ ID NO:3  NSLAISVMNQWPGVKLRVTEGWDEDGHHSE
```

Specific mutations may be designated according to the affected hedgehog gene product, *i.e.* [\*his SHH], [\*his DHH] and [\*his IHH], by the specific amino acid substitution, *i.e.* [his→tyr HH]; [ $\Delta$  his HH], *etc.*, or both, *i.e.* [his→tyr SHH]. Mutations may also be specified in terms of the DNA sequence. Proteins of particular interest have a substitution  
 5 of tyrosine, phenylalanine or tryptophan for [\*his HH]. The substitution of other non-polar or non-charged amino acids at this position are also of interest, *i.e.* alanine, valine, leucine, isoleucine, proline, methionine, glycine, serine, cysteine, asparagine and glutamine.

The mutated form of *sonic hedgehog*, [his→tyr SHH; SEQ ID NO:4], is  
 10 associated with oncogenic transformation of human cells. This is demonstrated by the presence of the mutated DNA sequence (C→T transition at nt. 548; SEQ ID NO:5) in a number of independently arising tumors. The spontaneously occurring nucleotide change encodes a tyrosine residue, but other codons can be engineered into the gene sequence, through conventional recombinant DNA technology, to provide proteins having a similar  
 15 phenotype. The high degree of homology between HH proteins in this region indicates a shared structure and function, with a similar phenotypic change resulting from this amino acid change in IHH and DHH. It is believed that this mutation alters the zinc hydrolase activity of the protein.

Other mutations of interest include an amino acid substitution of the  
 20 methionine found at residue 114 of SHH, herein referred to as a [\*met HH] mutation, sequences shown below, with the methionine residue underlined, and asterisks denoting identity between two sequences. The known HH proteins have a high degree of sequence identity in this region. Of particular interest is a change from met→ile, *e.g.* [met→ile SHH]. The gene, SEQ ID NO:10, encodes this mutated protein, SEQ ID NO:9, and was  
 25 found in the germline of a basal cell carcinoma patient.

```
(DHH) SEQ ID NO:6  RVNALAIAVMNMWPGVRLRVTEGWDED
                    *****
(SHH) SEQ ID NO:7  KLNALAISVMNQWPGVKLRVTEGWDED
                    ***
30 (IHH) SEQ ID NO:8  RLNSLAISVMNQWPGVKLRVTEGWDED
```

Nucleic Acid Compositions

The human [his→tyr SHH] amino acid sequence is provided (SEQ ID NO:4), and the encoding gene, isolated from human tumors, as (SEQ ID NO:5). In order to identify the subject oncogenic HH mutations, exonic primers from the published sequence  
5 data were used to isolate *SHH* genomic clones. Sequence data from the genomic clones was used to generate specific primers. These primers were used to amplify genomic DNA from primary tumors, including breast carcinomas, basal cell carcinomas and medulloblastomas. The PCR products were screened for mutations using single strand conformation polymorphism (SSCP) analysis. The specific mutation found in SEQ ID  
10 NO:5 was isolated from four independent tumors.

DNA encoding a [\*his HH] protein may be cDNA or genomic DNA or a fragment thereof that encompasses the altered [\*his] residue. As known in the art, cDNA sequences have the arrangement of exons found in processed mRNA, forming a continuous open reading frame, while genomic sequences may have introns interrupting the open  
15 reading frame. The term "[\*his HH] gene" shall be intended to mean the open reading frame encoding such specific HH polypeptides, as well as adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 1 kb beyond the coding region, in either direction.

Genomic sequences of interest comprise the nucleic acids present between the  
20 initiation codon and the stop codon, including all of the introns that are normally present in a native chromosome. It may include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, *etc.*, including about 1 kb of flanking genomic DNA at either the 5' or 3' end of the coding region. The genomic DNA may be isolated  
25 as a fragment of 50 kbp or smaller; and substantially free of flanking chromosomal sequence.

The nucleic acid compositions of the subject invention encode all or a part of the subject polypeptides. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction  
30 enzyme digestion, by PCR amplification, *etc.* For the most part, DNA fragments will be at least about 25 nt in length, usually at least about 30 nt, more usually at least about 50 nt. For use in amplification reactions, such as PCR, a pair of primers will be used. The exact



composition of the primer sequences is not critical to the invention, but for most applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to chose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms  
5 for the selection of primer sequences are generally known, and are available in commercial software packages. Amplification primers hybridize to complementary strands of DNA, and will prime towards each other.

The subject [\*his HH] genes are isolated and obtained in substantial purity, generally as other than an intact mammalian chromosome. Usually, the DNA will be  
10 obtained substantially free of other nucleic acid sequences that do not include a *hh* sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more nucleotides with which it is not normally associated on a naturally occurring chromosome.

The subject nucleic acids may be used to identify expression of the gene in a  
15 biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, as genomic DNA or RNA, is well-established in the literature and does not require elaboration here. Conveniently, a biological specimen is used as a source of mRNA. The mRNA may be amplified by RT-PCR, using reverse transcriptase to form a complementary DNA strand, followed by polymerase chain reaction amplification using  
20 primers specific for the subject DNA sequences. Alternatively, the mRNA sample is separated by gel electrophoresis, transferred to a suitable support, *e.g.* nitrocellulose and then probed with a fragment of the subject DNA as a probe. Other techniques may also find use. Detection of mRNA having the subject sequence is indicative of *hedgehog* gene expression in the sample.

25

#### Synthesis of HH Protein

The subject nucleic acid compositions may be employed for producing [\*his HH] protein, or fragments thereof that encompass a [\*his] mutation. For expression, an expression cassette may be employed, providing for a transcriptional and translational initiation region, which may be inducible or constitutive, the coding region under the  
30 transcriptional control of the transcriptional initiation region, and a transcriptional and

translational termination region. Various transcriptional initiation regions may be employed which are functional in the expression host.

The peptide may be expressed in prokaryotes or eukaryotes in accordance with conventional ways, depending upon the purpose for expression. For large scale production  
5 of the protein, a unicellular organism or cells of a higher organism, *e.g.* eukaryotes such as vertebrates, particularly mammals, may be used as the expression host, such as *E. coli*, *B. subtilis*, *S. cerevisiae*, and the like. In many situations, it may be desirable to express the subject *hedgehog* gene in a mammalian host, whereby the *hedgehog* gene product will be glycosylated, and secreted.

10 With the availability of the protein in large amounts by employing an expression host, the protein may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified protein will generally be at least about 80% pure,  
15 preferably at least about 90% pure, and may be up to and including 100% pure. By pure is intended free of other proteins, as well as of cellular debris.

The polypeptide is used for the production of antibodies, where short fragments provide for antibodies specific for the particular polypeptide, and larger fragments allow for the production of antibodies over the surface of the protein.  
20 Antibodies may be raised to the normal or oncogenic forms. Of particular interest are antibodies that specifically recognize the mutated form of the protein, *i.e.* do not bind to the normal form. Antibodies may be raised to isolated peptides corresponding to these mutations, or to the native protein, *e.g.* by immunization with cells expressing HH, immunization with liposomes containing HH, *etc.* Such antibodies are useful in therapy  
25 and diagnosis, *e.g.* typing and staging of human carcinomas.

Antibodies are prepared in accordance with conventional ways, where the expressed polypeptide or protein is used as an immunogen, by itself or conjugated to known immunogenic carriers, *e.g.* KLH, pre-S HBsAg, other viral or eukaryotic proteins, or the like. Various adjuvants may be employed, with a series of injections, as  
30 appropriate. For monoclonal antibodies, after one or more booster injections, the spleen is isolated, the splenocytes immortalized, and then screened for high affinity antibody

binding. The immortalized cells, *e.g.* hybridomas, producing the desired antibodies may then be expanded. For further description, see *Monoclonal Antibodies: A Laboratory Manual*, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. If desired, the mRNA encoding the heavy and light chains may be  
5 isolated and mutagenized by cloning in *E. coli*, and the heavy and light chains mixed to further enhance the affinity of the antibody. Alternatives to *in vivo* immunization as a method of raising antibodies include binding to phage "display" libraries, usually in conjunction with *in vitro* affinity maturation.

#### Detection of HH Associated Tumors

10 DNA from a patient having a tumor suspected of association with aberrant HH signaling is analyzed for the presence of an oncogenic mutation in a *hh* gene, *e.g.* a [\*his HH] mutation. Genetic characterization of sporadic tumors generally requires analysis of tumor cell DNA or RNA, conveniently with a biopsy sample. The nucleic acids are screened for the presence of an oncogenic mutation, as compared to a normal sequence  
15 (previously referenced). Sporadic tumors associated with altered HH signaling include basal cell carcinomas; melanomas; squamous cell carcinomas; breast carcinomas; transitional bladder cell carcinoma; meningiomas; medullomas; fibromas of the heart and ovary; carcinomas of the lung, colon, ovary, kidney and esophagus; other carcinomas of the gut; *etc.*

20 A number of methods are available for analyzing genomic DNA sequences. Where large amounts of DNA are available, the genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis, or amplified by conventional techniques, such as the polymerase chain reaction (PCR). The use of the polymerase chain reaction is described in Saiki, *et al.* (1985) *Science* 239:487, and a review of current techniques may be found in  
25 Sambrook, *et al.* *Molecular Cloning: A Laboratory Manual*, CSH Press 1989, pp.14.2-14.33.

A detectable label may be included in the amplification reaction. Suitable labels include fluorochromes, *e.g.* fluorescein isothiocyanate (FITC), rhodamine, Texas  
30 Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-

4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6- carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5- carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, *e.g.*  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ; *etc.* The label may be a two stage system, where the amplified DNA is conjugated to biotin, 5 haptens, *etc.* having a high affinity binding partner, *e.g.* avidin, specific antibodies, *etc.*, where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The amplified or cloned fragment may be sequenced by dideoxy or other 10 methods, and the sequence of bases compared to the normal *HH* sequence. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, *etc.* Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices are used to detect conformational changes created by DNA sequence variation as alterations in 15 electrophoretic mobility. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilised on a solid support, as described in WO 95/11995, may also be used as a means of detecting the presence of variant sequences. Alternatively, where an oncogenic mutation creates or destroys a recognition site for a restriction endonuclease, the fragment is digested with that endonuclease, and the product 20 size fractionated to determine whether the fragment was digested. Fractionation is performed by gel electrophoresis, particularly on acrylamide or agarose gels.

Analysis of tumor cells for the presence of aberrant *HH* proteins may be performed by immunoassay. A sample is taken from a patient suspected of having a *HH*-associated tumor. Samples, as used herein, include biological fluids such as blood, 25 cerebrospinal fluid, tears, saliva, lymph, dialysis fluid and the like; organ or tissue culture derived fluids; and fluids extracted from physiological tissues. Also included in the term are derivatives and fractions of such fluids. Biopsy samples are of particular interest, *e.g.* skin lesions, organ tissue fragments, *etc.* Where metastasis is suspected, blood samples may be preferred. The number of cells in a sample will generally be at least about  $10^2$ , 30 usually at least  $10^4$ , more usually at least about  $10^5$ . The cells may be dissociated, in the case of solid tissues, or tissue sections may be analyzed. Alternatively a lysate of the cells may be prepared.

Diagnosis may be performed by a number of methods. The different methods all determine the presence of abnormal HH in patient cells suspected of having an oncogenic mutation. For example, detection may utilize staining of intact cells or histological sections, performed in accordance with conventional methods. The antibodies  
5 of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemilumescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well-known in the art. For example, the primary antibody may  
10 be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, *etc.*

15 An alternative method for diagnosis depends on the *in vitro* detection of binding between antibodies and HH in a cell lysate, supernatant or other fluid into which the tumor cells may secrete active HH protein. Measuring the concentration of HH binding in a sample or fraction thereof may be accomplished by a variety of specific assays. A conventional sandwich type assay may be used. For example, a sandwich assay  
20 may first attach HH-specific antibodies to an insoluble surface or support. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which polypeptides can be  
25 bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, *e.g.* magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (*e.g.* polystyrene), polysaccharides, nylon or  
30 nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Patient sample lysates or supernatants are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing antibodies. Preferably, a series of standards, containing known concentrations of normal and/or abnormal HH is assayed in parallel with the samples or aliquots thereof to serve as  
5 controls. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for binding, generally, from about 0.1 to 3 hr is sufficient. After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From  
10 one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound proteins present in the sample.

After washing, a solution containing a second, detecting antibody is applied. The antibody will bind the immobilized HH with sufficient specificity such that it can be distinguished from other components present. The second antibodies may be labeled to  
15 facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement of second receptor binding include radiolabels, such as  $^3\text{H}$  or  $^{125}\text{I}$ , fluorescers, dyes, beads, chemiluminescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred embodiment,  
20 the antibodies are labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. The  
25 incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second binding step, the insoluble support is again washed free of non-specifically bound material. The signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme  
30 substrate is provided so a detectable product is formed.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots

may be performed on protein gels or protein spots on filters, using a detection system specific for HH as desired, conveniently using a labeling method as described for the sandwich assay.

#### Down-Regulation of HH Expression

5           Antisense molecules specific for the oncogenic HH genes, *e.g.* [<sup>3</sup>H-HH], [<sup>3</sup>met HH], are used to down-regulate expression in cells suspected or shown to have [<sup>3</sup>H-HH] associated tumors. Administration of the antisense molecules has the effect of decreasing the oncogenic HH activity. The anti-sense reagent may be antisense oligonucleotides (ODN), particularly synthetic ODN having chemical modifications from  
10 native nucleic acids, or nucleic acid constructs that express such anti-sense molecules as RNA. The antisense sequence is complementary to the mRNA of the targeted HH gene, and inhibits expression of the targeted gene products. Antisense molecules inhibit gene expression through various mechanisms, *e.g.* by reducing the amount of mRNA available for translation, through activation of RNase H, or steric hindrance. One or a combination  
15 of antisense molecules may be administered, where a combination may comprise multiple different sequences.

          Antisense molecules may be produced by expression of all or a part of the target gene sequence in an appropriate vector, where the vector is introduced and expressed in the targeted tumor cells. The transcriptional initiation will be oriented such  
20 that the antisense strand is produced as an RNA molecule. The anti-sense RNA hybridizes with the endogenous sense strand mRNA, thereby blocking expression of the targeted gene. The native transcriptional initiation region, or an exogenous transcriptional initiation region may be employed. The promoter may be introduced by recombinant methods *in vitro*, or as the result of homologous integration of the sequence into a  
25 chromosome. Many strong promoters are known in the art, including the  $\beta$ -actin promoter, SV40 early and late promoters, human cytomegalovirus promoter, retroviral LTRs, methallothionein responsive element (MRE) and tetracycline-inducible promoter constructs, *etc.*

          Transcription vectors generally have convenient restriction sites located near  
30 the promoter sequence to provide for the insertion of nucleic acid sequences.

Transcription cassettes may be prepared comprising a transcription initiation region, the

target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, *e.g.* plasmid; retrovirus, *e.g.* lentivirus; adenovirus; and the like, where the vectors are able to transiently or stably be maintained in the tumor cells, usually for a period of at least about  
5 one day, more usually for a period of at least about several days to several weeks.

Alternatively, the antisense molecule is a synthetic oligonucleotide. Antisense oligonucleotides will generally be at least about 7, usually at least about 12, more usually at least about 20 nucleotides in length, and not more than about 500, usually not more than about 50, more usually not more than about 35 nucleotides in length, where the length is  
10 governed by efficiency of inhibition, specificity, including absence of cross-reactivity, and the like. It has been found that short oligonucleotides, of from 7 to 8 bases in length, can be strong and selective inhibitors of gene expression (see Wagner *et al.* (1996) Nature Biotechnology 14:840-844).

A specific region or regions of the endogenous sense strand mRNA sequence  
15 is chosen to be complemented by the antisense sequence, preferably encompassing the [\*his HH] mutation. Selection of a specific sequence for the oligonucleotide may use an empirical method, where several candidate sequences are assayed for inhibition of expression of the target gene in an *in vitro* or animal model. A combination of sequences may also be used, where several regions of the mRNA sequence are selected for antisense  
20 complementation.

Antisense oligonucleotides may be chemically synthesized by methods known in the art (see Wagner *et al.* (1993) *supra.* and Milligan *et al.*, *supra.*) Preferred oligonucleotides are chemically modified from the native phosphodiester structure, in order to increase their intracellular stability and binding affinity. A number of such  
25 modifications have been described in the literature, which alter the chemistry of the backbone, sugars or heterocyclic bases.

Among useful changes in the backbone chemistry are phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulfur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate  
30 derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH<sub>2</sub>-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire ribose phosphodiester backbone with a peptide linkage.



Sugar modifications are also used to enhance stability and affinity. The  $\alpha$ -anomer of deoxyribose may be used, where the base is inverted with respect to the natural  $\beta$ -anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity.

5           Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown to increase affinity and biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

10           Oligonucleotides may additionally comprise a targeting moiety that enhances uptake of the molecule by tumor cells. The targeting moiety is a specific binding molecule, *e.g.* an antibody or fragment thereof that recognizes molecules present on the surface of tumor cells. Bispecific antibodies, chimeric antibodies and single chain antibodies are known in the art. Suitably prepared non-human antibodies can be  
15 humanized in various ways. Linkage between the oligonucleotide and targeting moiety may use any conventional method, for example by disulfide, amide or thioether bonds, depending on the chemistry of the oligonucleotide backbone. Preferably the linkage will be cleaved inside the cell to liberate the oligonucleotide.

Oligonucleotides can be conjugated to hydrophobic residues, *e.g.* cholesterol,  
20 to protect from nucleases and to improve transport across cell membranes. Alternatively, conjugation to poly-L-lysine or other polyamines may also enhance delivery to the cell. A further modification that can be made is the addition of an intercalating component, such as acridine, capable of intercalating into the target mRNA and stabilizing the resultant hybrid. Antisense oligonucleotides may be transfected in combination with an enzyme(s)  
25 that will degrade antisense-mRNA complexes in the cell, *e.g.* RNase H. Any protein or enzyme that can preferentially degrade or sequester the antisense-mRNA duplex may be similarly useful.

As an alternative to anti-sense inhibitors, catalytic nucleic acid compounds, *e.g.* ribozymes, anti-sense conjugates, *etc.* may be used to inhibit gene expression.  
30 Ribozymes may be synthesized *in vitro* and administered to the patient, or may be encoded on an expression vector, from which the ribozyme is synthesized in the targeted cell (for example, see International Patent Application WO 95/23225, and Beigelman et al. (1995)

Nucl. Acids Res 23:4434-42). Examples of oligonucleotides with catalytic activity are described in WO 9506764. Conjugates of anti-sense ODN with a metal complex, *e.g.* terpyridylCu(II), capable of mediating mRNA hydrolysis are described in Bashkin *et al.* (1995) Appl Biochem Biotechnol 54:43-56

- 5           The antisense molecules and/or other inhibitory agents are administered by contact with the tumor cells under conditions that permit entry. The molecules may be provided in solution or in any other pharmacologically suitable form for administration, such as a liposome suspension. There are many delivery methods known in the art for enhancing the uptake of nucleic acids by cells. Useful delivery systems include Sendai
- 10 virus-liposome delivery systems (see Rapaport and Shai (1994) J. Biol. Chem. 269:15124-15131), cationic liposomes, polymeric delivery gels or matrices, porous balloon catheters (as disclosed by Shi *et al.* (1994) Circulation 90:955-951; and Shi *et al.* (1994) Gene Therapy 1:408-414), retrovirus expression vectors, and the like.

- The use of liposomes as a delivery vehicle is one method of interest. The
- 15 liposomes fuse with the cells of the target site and deliver the contents of the lumen intracellularly. The liposomes are maintained in contact with the cells for sufficient time for fusion, using various means to maintain contact, such as isolation, binding agents, and the like. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus, etc. The lipids may be any
- 20 useful combination of known liposome forming lipids, including cationic lipids, such as phosphatidylcholine. The remaining lipid will normally be neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like.

- For preparing the liposomes, the procedure described by Kato *et al.* (1991) J. Biol. Chem. 266:3361 may be used. Briefly, the lipids and lumen composition containing
- 25 the nucleic acids are combined in an appropriate aqueous medium, conveniently a saline medium where the total solids will be in the range of about 1-10 weight percent. After intense agitation for short periods of time, from about 5-60 sec., the tube is placed in a warm water bath, from about 25-40°C and this cycle repeated from about 5-10 times. The composition is then sonicated for a convenient period of time, generally from about
- 30 1-10 sec. and may be further agitated by vortexing. The volume is then expanded by adding aqueous medium, generally increasing the volume by about from 1-2 fold, followed

by shaking and cooling. This method allows for the incorporation into the lumen of high molecular weight molecules, particularly nucleic acids of one kbp or more. In this way oligonucleotides, plasmids, large genes, chromosomal fragments, viruses or viral segments may be introduced into cells efficiently.

- 5           The therapeutic agents are administered at a dose effective to reduce expression level of [\*his HH] at least about 50%, more usually at least 80%, and preferably to substantially undetectable levels.

#### Therapeutic Use of HH Protein

The subject recombinantly produced HH protein may be therapeutically  
10 administered in conditions characterized by inadequate HH signaling pathway activity, or where increased HH signaling is desirable. Examples of such uses are provided in the published International Patent Application WO95/18856, herein incorporated by reference. Hedgehog biological activity includes the ability to induce or otherwise modulate formation and differentiation of various tissues, including the head, limbs, lungs, central  
15 nervous system or mesodermal patterning of embryos. Proliferation is also modulated by HH in a number of tissues. Such modulation may be achieved in *in vitro* or *in vivo* situations. For example, wound healing, bone formation, the treatment of hypoproliferative or hyperproliferative skin disorders, induction of differentiation, are affected by administration of the subject proteins.

- 20           HH is able to regulate neurogenesis, such a motor neuron inducing activity, a neuronal differentiation inducing activity or a neuronal survival promoting activity. HH also regulates organogenesis and induction of stem cell or germ cell differentiation, including the ability to induce chondrocytes or an involvement in spermatogenesis. The treatment of arthritis, *e.g.* osteoarthritis, rheumatoid arthritis, *etc.* may benefit from  
25 administration of the subject proteins, and subsequent induction of chondrocytes and cartilage formation. HH is able to regulate the growth of hair by modulating the growth of cells in the hair sheath, and can be used therapeutically for this purpose.

Administration of HH induces expression of secondary signaling molecules, such as members of TGF beta family, bone morphogenetic proteins, and members of the  
30 fibroblast growth factor family.

Many neurological disorders are associated with degeneration of discrete populations of neuronal elements and may be treated with the subject HH proteins. Specific disorders include traumatic injury, injury resulting from ischemia resulting from stroke, damage resulting from inflammation and/or infection of the nervous system, Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, spinocerebellar degenerations, and chronic immunological diseases of the central nervous system, *e.g.* multiple sclerosis. The subject HH proteins are also useful in treating autonomic disorders of the peripheral nervous system, such as tachycardia or atrial cardiac arrhythmias arising from a degenerative condition of the nerves innervating the striated muscles of the heart.

Also of interest are *in vitro* uses, where HH is added to specific cell cultures, *e.g.* neural progenitor cells, which can terminally differentiate into neurons and glia. HH sustains the reproduction of such cells in culture, in combination with appropriate culture medium, as known in the art.

The host may be treated with intact [<sup>35</sup>S-HH] protein, or an active fragment thereof, particularly a cleaved fragment as generated by normal processing. Desirably, the peptides will not induce an immune response, particularly an antibody response. Xenogeneic analogs may be screened for their ability provide a therapeutic effect without raising an immune response.

Various methods for administration may be employed. The polypeptide formulation may be given orally, or may be injected intravascularly, subcutaneously, peritoneally, *etc.* The dosage of the therapeutic formulation will vary widely, depending upon the nature of the disease, the frequency of administration, the manner of administration, the clearance of the agent from the host, and the like. The initial dose may be larger, followed by smaller maintenance doses. The dose may be administered as infrequently as weekly or biweekly, or fractionated into smaller doses and administered daily, semi-weekly, *etc.* to maintain an effective dosage level. In many cases, oral administration will require a higher dose than if administered intravenously. The amide bonds, as well as the amino and carboxy termini, may be modified for greater stability on oral administration.

The subject peptides may be prepared as formulations at a pharmacologically effective dose in pharmaceutically acceptable media, for example normal saline, PBS, *etc.*

The additives may include bactericidal agents, stabilizers, buffers, or the like. In order to enhance the half-life of the subject peptide or subject peptide conjugates, the peptides may be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or another conventional technique may be employed that provides for an extended lifetime of the peptides.

The peptides may be administered as a combination therapy with other pharmacologically active agents. The additional drugs may be administered separately or in conjunction with the peptide compositions, and may be formulated in the same formulation.

#### 10 Genetically Modified Cells and Animals

The subject nucleic acids can be used to generate transgenic animals or site specific gene modifications in cell lines. Transgenic animals may be made through homologous recombination. Alternatively, a nucleic acid construct is randomly integrated into the genome. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. The modified cells or animals are useful in the study of *hedgehog* function and regulation. A detectable marker, such as *lac Z* may be introduced into the *hedgehog* locus, where upregulation of *hedgehog* expression will result in an easily detected change in phenotype.

DNA constructs for homologous recombination will comprise at least a portion of a [*\*his HH*] gene with the desired genetic modification, and will include regions of homology to the target locus. DNA constructs for random integration need not include regions of homology to mediate recombination. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown *et al.* (1990) Methods in Enzymology 185:527-537.

For embryonic stem (ES) cells, an ES cell line may be employed, or ES cells may be obtained freshly from a host, *e.g.* mouse, rat, guinea pig, *etc.* Such cells are grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibiting factor (LIF). When ES cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer

in an appropriate medium. Cells containing the construct may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination or integration of the construct. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females. The ES cells are trypsinized, and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected.

The chimeric animals are screened for the presence of the modified gene and males and females having the modification are mated to produce homozygous progeny. The transgenic animals may be any non-human mammal, such as laboratory animals, domestic animals, etc. The transgenic animals may be used in functional studies, drug screening, *etc.*, *e.g.* to determine the effect of a candidate drug on a HH-associated tumor.

#### Drug Screening Assays

Drug screening identifies agents that provide a replacement for HH function in abnormal cells. The role of HH as an oncogene indicates that agents which antagonize its function will inhibit the process of oncogenesis. Conversely, agents that mimic HH function may stimulate controlled growth and healing. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transporter function, *etc.*

The term "agent" as used herein describes any molecule, *e.g.* protein or pharmaceutical, with the capability of altering or mimicking the physiological function of *hedgehog*. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, *i.e.* at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemilumescers, enzymes, specific binding molecules, particles, *e.g.* magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin *etc.* For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, *e.g.* albumin, detergents, *etc.* that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors,

nuclease inhibitors, anti-microbial agents, *etc.* may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of cancer or developmental abnormalities attributable to a defect in *hedgehog* function. The compounds may also be used to enhance *hedgehog* function in wound healing, aging, *etc.* The inhibitory agents may be administered in a variety of ways, orally, topically, parenterally *e.g.* subcutaneously, intraperitoneally, by viral infection, intravascularly, *etc.* Topical treatments are of particular interest. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt. %.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to the numbers used (*e.g.* amounts, temperature, concentrations, *etc.*) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.



## EXPERIMENTAL

Identification of Mutations in Sonic hedgehog in Human Cancers

DNA was screened from a number of human primary tumors for mutations in the *SONIC HEDGEHOG (SHH)* gene. Primers specific for the exons (coding region  
 5 sequences) were derived from the published gene sequence (previously referenced).

BAC clones were obtained by PCR screening of human BAC DNA pools from Research Genetics, Inc. (Huntsville, AL) (Shizuya *et al.* (1992) P.N.A.S. 89:8794-8797. Briefly, DNA from BAC pools was used as a template in PCR with a specific exon 2 primer pair, SEQ ID NO:11; ACCGAGGGC TGGGACGAA GATGGC and SEQ ID  
 10 NO:12; GCGAGCCAGCATGCCGTACTTGCTG. Only pools comprising a BAC that contains the sequence tag defined by the primer pair will yield an amplification product. This process is continued until a single positive BAC can be identified. A positive clone, BAC270A17, was digested with restriction enzymes and ligated with vectorette linkers, as described in Riley *et al.* (1990) Nucl. Acids Res. 18:2887-2890.

15 Exon intron boundaries were determined for the three *SHH* exons by sequencing PCR products amplified using the universal vectorette primer and *SHH* cDNA primers derived from the published sequences. The exon 2-intron 3 boundary was difficult to sequence, and so a primer from the 3' end of exon 2 was used. The following primers were used to amplify genomic *SHH*:

20	exon 1;	(SEQ ID NO:13) CCGCCGCGCGCACTCG
		(SEQ ID NO:14) AAGGAGCGGGTGAAATCACC
	exon 2;	(SEQ ID NO:15) TAACGTGTCCGTCGGTGGG
		(SEQ ID NO:16) TGCTTTCACCGAGCAGTGG
	exon 3	(SEQ ID NO:17) CCTCCTCCCCGAGACGC
25		(SEQ ID NO:18) GGCCCCCTCCCGCGCC

These primers were used to amplify genomic DNA from primary human tumors: 34 independent basal cell carcinomas, 14 medulloblastomas and 6 breast carcinomas. PCR was performed using 1-100 ng genomic DNA, primers and AmpliTaq™ (Perkin Elmer, CA) under the following conditions: 95°C 1 min denaturation, 94°C 20  
 30 sec, 58°C 20 sec, 72°C 30 sec for 30 cycles followed by 72°C 2 min.

The amplification product was used in single strand conformational polymorphism (SSCP) analysis against the wild-type *SHH* gene, performed according to

the method of Chen *et al.* (1995) J. Invest Dermatol, 105:629-632. No polymorphisms were found in exons 1 and 3.

SSCP abnormalities were identified in 2 of the basal cell carcinomas, 1 of the medulloblastomas and one breast carcinoma. The aberrant SSCP bands were identical in one of the basal cell carcinomas and the two other positive tumors. No aberrantly migrating bands were found on SSCP analysis of SHH exon 2 in blood cell DNA from 100 normal persons, or from the three individuals from which the tumor cells were derived, or in exons 1 and 3. Sequencing identified the same mutation in all three tumors, a C to T transition at bp 548 (SEQ ID NO:5), predicted to cause the mutation of SEQ ID NO:4.

A second basal cell carcinoma also had an abnormally migrating band on SSCP analysis of exon 2, but the same abnormal product was also present in blood cell DNA. This is a G to A substitution at bp 493 (SEQ ID NO:9), encoding the mutated protein of SEQ ID NO:10. This individual had had at least six other basal cell carcinomas, her age at onset of the studied basal cell carcinoma was approximately 40. DNA was not available from other family members, none of whom is known to have developmental abnormalities or cancers, other than small basal cell carcinomas in both of her parents on sun exposed areas after age 60. The studied carcinoma was unusually large, involving most of one cheek, the eyelids and ear, with invasion and destruction of the facial nerve. There were no phenotypic abnormalities associated with basal cell nevus syndrome present.

SSCP analysis of the human *PATCHED* gene (*PTC*) in these four tumors identified a missense mutation in one allele of the breast carcinoma cells. Analysis of an intragenic polymorphism and direct sequencing of the mutant allele indicated a retention of both *PTC* alleles in this tumor

SHH expression in normal human epidermal keratinocytes was not detectable by Northern blot analysis under conditions in which *PTC* gene expression was easily detected, irrespective of whether the cells were cultured in low or high calcium media (0.08 or 1.2 mM). However, SHH primers did amplify a product by reverse transcriptase PCR of RNA from cultured normal human epidermal keratinocytes, and sequencing confirmed the identity of the product as authentic SHH.

DNA was extracted from keratinocytes cultured at 0.07 mM calcium using the Pharmacia Quick Prep mRNA kit. First strand cDNA was synthesized with a Gene Amp

kit (Perkin Elmer) using an oligo-dT primer. cDNA was amplified by three-stage nesting using the following primers:

Stage 1, (SEQ ID NO:19) CCGCCGCGCGCACTCG and (SEQ ID NO:20) GGCCCCCTCCCGCGCC; Stage 2, (SEQ ID NO:22) TGCCACCGGGCAGGGG and  
5 (SEQ ID NO:21) GTCCTTCACCAGCTTGGTG; Stage 3, (SEQ ID NO:22) and (SEQ ID NO:23) TCAGCCTCCCATTGCGCCA.

The above results indicate that a single somatic mutation is found in cancers arising from three different tissues in independent patients. This finding indicates the importance of this mutation in carcinogenesis.

10 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of  
15 prior invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or  
20 scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: The Regents of the University of California
- (ii) TITLE OF INVENTION: Mutant Human Hedgehog Gene
- (iii) NUMBER OF SEQUENCES: 23
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Bozicevic & Reed LLP
  - (B) STREET: 285 Hamilton Avenue, Suite 200
  - (C) CITY: Palo Alto
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 94301
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: unknown
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Sherwood, Pamela J
  - (B) REGISTRATION NUMBER: 36,677
  - (C) REFERENCE/DOCKET NUMBER: 9076/067WO1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (415) 327-3400
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

WO 98/21227

PCT/US97/20227

Asn	Ala	Leu	Ala	Ile	Ala	Val	Met	Asn	Met	Trp	Pro	Gly	Val	Arg	Leu
1				5						10				15	
Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ala	Gln		
			20					25					30		

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn	Ala	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu
1				5						10				15	
Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu		
			20					25					30		

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn	Ser	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu
1				5						10				15	
Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu		
			20					25					30		

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 462 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
1           5           10           15

Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
20           25           30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35           40           45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50           55           60

Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65           70           75           80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
85           90           95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
100          105          110

Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
115          120          125

Asp Glu Asp Gly Tyr His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
130          135          140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
145          150          155          160

Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
165          170          175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
180          185          190

Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
195          200          205

Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
210          215          220

Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
225          230          235          240

Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
245          250          255

Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
260          265          270

Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
275          280          285

```

Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu  
 290 295 300

Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu  
 305 310 315 320

Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr  
 325 330 335

Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly  
 340 345 350

Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu  
 355 360 365

Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His  
 370 375 380

Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp  
 385 390 395 400

Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr  
 405 410 415

Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile  
 420 425 430

His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp  
 435 440 445

Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser  
 450 455 460

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1576 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGAGGCAGC CAGCGAGGGA GAGAGCGAGC GGGCGAGCCG GAGCGAGGAA GGGAAAGCGC 60

AAGAGAGAGC GCACACGCAC ACACCCGCCG CGCGCACTCG CGCCCGGACC CGCACGGGGA 120

CAGCTCGGAA GTCATCAGTT CCATGGGCGA GATGCTGCTG CTGGCGAGAT GTCTGCTGCT 180

AGTCCTCGTC TCCTCGCTGC TGGTATGCTC GGGACTGGCG TGCGGACCGG GCAGGGGGTT 240

CGGGAAGAGG AGGCACCCCA AAAAGCTGAC CCCTTTAGCC TACAAGCAGT TTATCCCCAA 300

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TGTGGCCGAG AAGACCCTAG GCGCCAGCGG AAGGTATGAA GGGAAGATCT CCAGAAACTC	360
CGAGCGATTT AAGGAACTCA CCCCCAATTA CAACCCCGAC ATCATATTTA AGGATGAAGA	420
AAACACCGGA GCGGACAGGC TGATGACTCA GAGGTGTAAG GACAAGTTGA ACGCTTTGGC	480
CATCTCGGTG ATGAACCACT GGCCAGGAGT GAAACTGCCG GTGACCGAGG GCTGGGACGA	540
AGATGGCTAC CACTCAGAGG AGTCTCTGCA CTACGAGGGC CGCGCAGTGG ACATCACCAC	600
GTCTGACCGC GACCGCAGCA AGTACGGCAT GCTGGCCCGC CTGGCGGTGG AGGCCGGCTT	660
CGACTGGGTG TACTACGAGT CCAAGGCACA TATCCACTGC TCGGTGAAAG CAGAGAACTC	720
GGTGGCGGCC AAATCGGGAG GCTGCTTCCC GGGCTCGGCC ACGGTGCACC TGGAGCAGGG	780
CGGCACCAAG CTGGTGAAGG ACCTGAGCCC CGGGGACCGC GTGCTGGCGG CGGACGACCA	840
GGGCCGGCTG CTCTACAGCG ACTTCCTCAC TTTCTGGAC CGCGACGACG GCGCCAAGAA	900
GGTCTTCTAC GTGATCGAGA CGCGGGAGCC GCGCGAGCGC CTGCTGCTCA CCGCCGCGCA	960
CCTGCTCTTT GTGGCGCCGC ACAACGACTC GGCCACCGGG GAGCCCGAGG CGTCTCGGG	1020
CTCGGGGCCG CCTTCCGGGG GCGCACTGGG GCCTCGGGCG CTGTTGCCA GCCGCGTGCG	1080
CCCCGGCCAG CGCGTGACG TGGTGGCCGA GCGTGACGGG GACCGCCGCG TCCTGCCCCG	1140
CGCTGTGCAC AGCGTGACCC TAAGCGAGGA GGCCGCGGGC GCCTACGCGC CGCTCACGGC	1200
CCAGGGCACC ATTCTCATCA ACCGGGTGCT GGCCTCGTGC TACGCGGTCA TCGAGGAGCA	1260
CAGCTGGGCG CACCGGGCCT TCGCGCCCTT CCGCCTGGCG CACGCGCTCC TGGCTGCACT	1320
GGCGCCCGCG CGCACGGACC GCGGCGGGGA CAGCGCGGCG GGGGACCGCG GGGGCGGCGG	1380
CGGCAGAGTA GCCCTAACCG CTCCAGGTGC TGCCGACGCT CCGGGTGCGG GGGCCACCGC	1440
GGGCATCCAC TGGTACTCGC AGCTGCTCTA CCAAATAGGC ACCTGGCTCC TGGACAGCGA	1500
GGCCCTGCAC CCGCTGGGCA TGGCGGTCAA GTCCAGCTGA AGCCGGGGGG CCGGGGGAGG	1560
GGCGCGGGAG GGGGCC	1576

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg	Val	Asn	Ala	Leu	Ala	Ile	Ala	Val	Met	Asn	Met	Trp	Pro	Gly	Val
1				5					10					15	
Arg	Leu	Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp					
				20				25							

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys	Leu	Asn	Ala	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val
1				5					10					15	
Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp					
				20				25							

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg	Leu	Asn	Ser	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val
1				5					10					15	
Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	Asp	Glu	Asp					
				20				25							

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 462 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1             5             10             15

Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20             25             30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
 35             40             45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
 50             55             60

Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65             70             75             80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
 85             90             95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
100            105            110

Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
115            120            125

Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
130            135            140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
145            150            155            160

Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
165            170            175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
180            185            190

Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
195            200            205

Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
210            215            220

Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
225            230            235            240

Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
245            250            255

Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
260            265            270

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Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Ala	Thr	Gly	Glu	Pro	Glu	Ala	Ser	
	275						280					285				
Ser	Gly	Ser	Gly	Pro	Pro	Ser	Gly	Gly	Ala	Leu	Gly	Pro	Arg	Ala	Leu	
	290					295					300					
Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val	Ala	Glu	
305					310					315					320	
Arg	Asp	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	Ala	Val	His	Ser	Val	Thr	
				325					330					335		
Leu	Ser	Glu	Glu	Ala	Ala	Gly	Ala	Tyr	Ala	Pro	Leu	Thr	Ala	Gln	Gly	
		340						345					350			
Thr	Ile	Leu	Ile	Asn	Arg	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Ile	Glu	
	355						360					365				
Glu	His	Ser	Trp	Ala	His	Arg	Ala	Phe	Ala	Pro	Phe	Arg	Leu	Ala	His	
	370					375					380					
Ala	Leu	Leu	Ala	Ala	Leu	Ala	Pro	Ala	Arg	Thr	Asp	Arg	Gly	Gly	Asp	
385					390					395					400	
Ser	Gly	Gly	Gly	Asp	Arg	Gly	Gly	Gly	Gly	Gly	Arg	Val	Ala	Leu	Thr	
				405					410					415		
Ala	Pro	Gly	Ala	Ala	Asp	Ala	Pro	Gly	Ala	Gly	Ala	Thr	Ala	Gly	Ile	
		420						425					430			
His	Trp	Tyr	Ser	Gln	Leu	Leu	Tyr	Gln	Ile	Gly	Thr	Trp	Leu	Leu	Asp	
	435						440					445				
Ser	Glu	Ala	Leu	His	Pro	Leu	Gly	Met	Ala	Val	Lys	Ser	Ser			
	450					455					460					

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1576 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCGAGGCAGC CAGCGAGGGA GAGAGCGAGC GGGCGAGCCG GAGCGAGGAA GGGAAAGCGC	60
AAGAGAGAGC GCACACGCAC ACACCCGCCG CGCGCACTCG CGCCCGGACC CGCACGGGGA	120
CAGCTCGGAA GTCATCAGTT CCATGGGCGA GATGCTGCTG CTGGCGAGAT GTCTGCTGCT	180

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AGTCCTCGTC TCCTCGCTGC TGGTATGCTC GGGACTGGCG TCGGACCGG GCAGGGGGTT	240
CGGGAAGAGG AGGCACCCCA AAAAGCTGAC CCCTTTAGCC TACAAGCAGT TTATCCCCAA	300
TGTGGCCGAG AAGACCCTAG GCGCCAGCGG AAGGTATGAA GGGAAGATCT CCAGAACTC	360
CGAGCGATTT AAGGAACTCA CCCCCAATTA CAACCCCGAC ATCATATTTA AGGATGAAGA	420
AAACACCGGA GCGGACAGGC TGATGACTCA GAGGTGTAAG GACAAGTTGA ACGCTTTGGC	480
CATCTCGGTG ATAAACCAGT GGCCAGGAGT GAAACTGCGG GTGACCGAGG GCTGGGACGA	540
AGATGGCCAC CACTCAGAGG AGTCTCTGCA CTACGAGGCG CGCGCAGTGG ACATCACCAC	600
GTCTGACCGC GACCGCAGCA AGTACGGCAT GCTGGCCCGC CTGGCGGTGG AGGCCGGCTT	660
CGACTGGGTG TACTACGAGT CCAAGGCACA TATCCACTGC TCGGTGAAAG CAGAGAACTC	720
GGTGGCGGCC AAATCGGGAG GCTGCTTCCC GGGCTCGGCC ACGGTGCACC TGGAGCAGGG	780
CGGCACCAAG CTGGTGAAGG ACCTGAGCCC CGGGGACCGC GTGCTGGCGG CGGACGACCA	840
GGGCCGGGTG CTCTACAGCG ACTTCCTCAC TTTCTGGAC CGCGACGACG GCGCCAAGAA	900
GGTCTTCTAC GTGATCGAGA CGCGGGAGCC GCGCGAGCGC CTGCTGCTCA CCGCCGCGCA	960
CCTGCTCTTT GTGGCGCCGC ACAACGACTC GGCCACCGGG GAGCCCGAGG CGTCCTCGGG	1020
CTCGGGGCGC CCTTCCGGGG GCGCACTGGG GCCTCGGGCG CTGTTGCGCA GCCGCGTGCG	1080
CCCCGGCCAG CGCGTGACG TGGTGGCCGA GCGTGACGGG GACCGCCGGC TCCTGCCCCG	1140
CGCTGTGCAC AGCGTGACCC TAAGCGAGGA GGCCGCGGGC GCCTACGCGC CGCTCACGGC	1200
CCAGGGCACC ATTCTCATCA ACCGGGTGCT GGCCTCGTGC TACGCGGTCA TCGAGGAGCA	1260
CAGCTGGGCG CACCGGGCCT TCGCGCCCTT CCGCCTGGCG CACGCGCTCC TGGCTGCACT	1320
GGCGCCCGCG CGCACGGACC GCGGCGGGGA CAGCGGCGGC GGGGACCGCG GGGGCGGCGG	1380
CGGCAGAGTA GCCCTAACCG CTCAGGTGC TGCCGACGCT CCGGGTGCGG GGGCCACCGC	1440
GGGCATCCAC TGGTACTCGC AGCTGCTCTA CCAAATAGGC ACCTGGCTCC TGGACAGCGA	1500
GGCCCTGCAC CCGCTGGGCA TGGCGGTCAA GTCCAGCTGA AGCCGGGGGG CCGGGGGAGG	1560
GGCGCGGGAG GGGGCC	1576

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACCGAGGGCT GGGACGAAGA TGGC

24

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGAGCCAGC ATGCCGTACT TGCTG

25

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGCCGCGCG CACTCG

16

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGGAGCGGG TGAAATCACC

20

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TAACGTGTCC GTCGGTGGG

19

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCTTTCACC GAGCAGTGG

19

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 17 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCTCCTCCCC GAGACGC

17

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGCCCCCTCC CGCGCC

16

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCGCCGCGCG CACTCG

16

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCCCCCTCC CGCGCC

16

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTCCTTCACC AGCTTGGTG

19

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TGCCCACCGG GCAGGGGG

18

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCAGCCTCCC ATTTGGCCGC CA

22



## WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding an oncogenic human HH protein, as other than an intact chromosome.
2. An isolated nucleic acid according to Claim 1, encoding a human [\*his  
5 HH] protein or a fragment of at least 18 nucleotides derived therefrom and encompassing said [\*his HH] mutation, as other than an intact chromosome.
3. An isolated nucleic acid according to Claim 1, encoding a human [\*met HH] protein or a fragment of at least 18 nucleotides derived therefrom and encompassing said [\*met HH] mutation, as other than an intact chromosome.
- 10 4. An isolated nucleic acid according to Claim 1, wherein said protein is selected from the group consisting of sonic hedgehog, indian hedgehog and desert hedgehog.
5. An isolated nucleic acid according to Claim 1, encoding a protein selected from the group consisting of human [his→tyr HH] and [met→ile HH].
- 15 6. An isolated nucleic acid according to Claim 5, wherein said protein is selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:9.
7. An isolated nucleic acid according to Claim 6, selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:10.
8. An expression cassette comprising a transcriptional initiation region, a  
20 nucleic acid according to Claim 1 under the transcriptional regulation of said transcriptional initiation region, and a transcriptional termination region.
9. A cell comprising an expression cassette according to Claim 8.
10. A cell according to Claim 9, wherein said cell is a mammalian cell.

11. A cell according to Claim 10, wherein said mammalian cell is human.
12. A method for producing oncogenic HH protein, said method comprising:  
growing a cell according to Claim 9, whereby said oncogenic HH protein is  
5 expressed; and  
isolating said oncogenic HH protein free of other proteins.
13. A purified polypeptide composition comprising at least 50 weight % of the protein present as an oncogenic HH protein.
14. A purified polypeptide composition according to Claim 13, wherein  
10 said protein is selected from the group consisting of sonic hedgehog, indian hedgehog, and desert hedgehog.
15. A purified polypeptide composition according to Claim 13, wherein said protein is selected from the group consisting of human [his→tyr HH] and [met→ile HH].
- 15 16. A purified polypeptide composition according to Claim 13, wherein said protein is selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:9.
17. A method for characterizing the phenotype of a tumor, the method comprising:  
20 detecting the presence of an oncogenic HH mutation in said tumor,  
wherein the presence of said oncogenic HH mutation indicates that said tumor is associated with aberrant HH signaling.
18. A method according to Claim 17, wherein said detecting step determines the presence of said oncogenic HH mutation in a nucleic acid, selected from the  
25 group consisting of genomic DNA and mRNA.

19. A method according to Claim 18, wherein said detecting step determines the presence of mutated [his→tyr SHH] protein.
20. An isolated antibody specific for a human oncogenic HH protein.
21. An isolated antibody according to Claim 18, wherein said antibody is a  
5 monoclonal antibody.
22. An assay device comprising:  
a support surface; and  
the antibody of claim 21 bound to said support surface.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20227

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 252.3, 254.11, 320.1, 325, 366; 530/350, 387.7, 387.9, 388.1; 536/23.1, 23.5; 935/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: APS, CAS ONLINE, MEDLINE

Keywords: hedgehog, sonic, desert, imdiam, vectors, genes, DNA, deoxyribo?, human, basal cell nevus, basal cell carcinoma

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO/9518856 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 13 July 1995. see whole document.	1-22
Y, P	ORO et al. Basal cell carcinomas in mice overexpressing sonic hedgehog. Science. 02 May 1997. Vol. 276. see whole document.	1-19
A	GAILANI et al. The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. Nature Genetics. September 1996. Vol. 14. see whole document.	1-22
A, P	HAMMERSCHMIDT et al. The world according to hedgehog. Trends in Genetics. January 1997. Vol. 13. No. 1. see whole document.	1-22

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 JANUARY 1998

Date of mailing of the international search report

18 FEB 1998

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20227

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	STONE et al. The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature. 14 November 1996. Vol. 384. see whole document.	1-22
A	OGURA et al. Evidence that Shh cooperates with retinoic acid inducible co-factor to establish ZPA-like activity. Development. February 1996. Vol. 122. see whole document.	1-22
A	HAHN et al. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell. 14 June 1996. Vol. 85. see whole document.	1-7
A, P	ROESSLER et al. Mutations in the human sonic hedgehog gene cause holoprosencephaly. Nature Genetics. November 1996. Vol. 14. see whole document.	1-7
X	MARIGO et al. Cloning, expression and chromosomal location of SHH and IHH: Two human homologues of the Drosophila segment polarity gene hedgehog. Genomics. 01 July 1995. Vol. 28. see whole document.	1, 4 and 8

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20227

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20227

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 21/04, 21/02; C07K 1/00, 16/00; C12N 1/14, 1/20, 5/00, 5/08, 15/00; C12P 21/06; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 252.3, 254.11, 320.1, 325, 366; 530/350, 387.7, 387.9, 388.1; 536/23.1, 23.5; 935/22

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-19, drawn to human hedgehog proteins, nucleic acids encoding human hedgehog proteins, vectors, transformed host cells and expression systems, thereof and a method of use.

Group II, claims 20-22, drawn to antibodies against human hedgehog proteins.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of the Group I invention is the specific amino acid sequence of the oncogenic mutant human hedgehog proteins and the specific nucleotide sequence of the nucleic acids encoding the human hedgehog proteins, whereas the special technical feature of the Group II invention is the antibodies against the oncogenic human hedgehog proteins. Since the special technical feature of the Group I invention is not present in the Group II claims, and the special technical feature of the Group II invention is not present in the Group I claims, unity of invention is lacking.